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Symposium on Microbial Insecticides

I. Bacterial Pathogens of Insects as Microbial Insecticides[1]

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INTRODUCTION

In a symposium on microbial insecticides, it is appropriate to recall that it was Agostino Bassi who, in 1838, first proposed that microorganisms might be used to control insects (67). About 100 years ago, Pasteur noted the presence of bacteria in diseased silkworms, and since that time a large number of associations between insects and bacteria have been reported (65, 66, 68, 72).

Classification of Entomogenous Bacteria

Bucher (16) proposed a useful classification of the entomogenous bacteria which has been widely adopted. In it, *obligate pathogens* are defined as those found associated with a specific insect disease; they have a narrow host range, are readily transmitted, require specialized conditions for growth, and in nature probably multiply only within the bodies of certain species of insects. The *facultative pathogens* are a group of bacteria that possess some mechanism for damaging or invading a susceptible tissue but are not obligate parasites; they are readily cultured in artificial media and are capable of multiplying within the gut of the host insect before invasion of the hemocoele. *Potential pathogens* are those that normally do not multiply in the gut but can do so in the hemocoele once they gain access to it; they grow readily on artificial media, and are not associated with a specific disease of specific insects.

Applying these criteria, there are many bacterial species that can be classed as insect pathogens but only a few of these have shown any promise as microbial insecticides; this has been discussed in a number of papers (3, 12, 26, 29, 30, 32, 33, 53, 67, 73, 81, 82). The factors that govern whether a pathogen is endemic or epidemic in a given population are exceedingly complex, and insofar as insects are concerned our knowledge is at best fragmentary (83).

Attributes of an Ideal Microbial Insecticide

Previous attempts to utilize bacteria as microbial insecticides have revealed certain attributes that materially affect successful use. First of all, the prospective pathogen should be virulent, at least to the extent that it consistently causes a disease serious enough to inhibit the competitive activity of the pest insect. Variations in virulence, when they occur, should not be so great as to affect materially a recommended dosage or require frequent assays. The pathogen should not be markedly sensitive to the environmental hazards to which it will be exposed (such as desiccation and sunlight), to the way in which it will be introduced (such as a spray or a dust), or to the suspending medium (oil, water, stickers, emulsifying agents) used. It should also be persistent, in the sense that it will remain viable or infectious until it gains access to the target insect. In general terms, it is preferable that the pathogen be rapid in its action, for it is the feeding activity of most agricultural and forest insects that makes them pest species; this is not a rigorous requirement however. It is important that

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the pathogen be fairly specific for the insect pest it is used against, and inactive against the host plant and useful insect species such as parasites and pollinators. It is of extreme importance that the pathogen be harmless, under the conditions of use, for vertebrates, and especially for mammals. Finally, it must be possible to produce the pathogen in quantity at an economically acceptable cost in a form that is practicable and aesthestically satisfactory. Taken as a whole, this is a rigorous set of requirements that excludes most isolates from consideration (3, 11, 12, 26, 32, 33, 53, 71, 83).

The known bacterial pathogens of insects are found principally in two families: the Enterobacteriaceae and the Bacillaceae; a few species occur in the Pseudomonadales (18, 45). The first of the bacteria to be considered for microbial control was identified by d'Herelle as *Coccobacillus acridiorum*. Early optimistic claims for its usefulness in reducing grasshopper populations have not been substantiated by subsequent attempts to use it. Bucher (14) reviewed all of this earlier work, and concluded that *C. acridiorum* is identified as Cloaca type A of the family Enterobacteriaceae, strains of which are wide-spread inhabitants of the gut of grasshoppers. In Bucher's opinion, it should not be classed as a true pathogen.

NONSPORULATING BACTERIAL PATHOGENS

Pseudomonas aeruginosa

It has been shown that *P. aeruginosa* (Schroeter) Migula also is pathogenic for the grasshoppers *Melanoplus bivittatus* (Say) and *Camnula pellucida* (Scudder). Bucher classified *P. aeruginosa* as a potential pathogen, that is, a species which does not normally multiply in the insect gut but can do so in the hemocoele from small inocula. Ruptures often occur in the grasshopper gut, and it may be that *P. aeruginosa* gains access to the insect hemocoele in this accidental way rather than as the result of some invasive mechanism. This species often causes disease in laboratory rearings of grasshoppers, but natural infection in field populations has never been demonstrated (15, 18, 19, 20, 77). It is known that *P. aeruginosa* is readily killed by drying and sunlight. This can be partially offset by use of a mixture of mucin, sucrose, and casein as a coating. A limited field trial of *P. aeruginosa* so protected, as a spray and in baits, failed to exert any useful level of control (76, 78).

Lysenko recently demonstrated in *P. aeruginosa* cultures the occurrence of an antigenic compound that is toxic for larvae of *Galleria mellonella* L. by injection (49, 50, 51, 52); it is not known whether this toxin plays a role in a natural infection. *P. aeruginosa* also produces a phospholipase (60); an enzyme of this type is known to be an important toxin in certain entomogenous strains of *Bacillus cereus* (37).

Serratia marcescens

S. marcescens Bizio is not nutritionally fastidious, grows in a wide *p*H range, is a facultative anaerobe, and is strongly proteolytic; therefore, one would expect it to be capable of multiplying in the gut of many insect species. Although most strains are pathogenic if injected into the hemocoele of insects, only a few cause disease if taken by mouth (18, 39, 58, 69). *S. marcescens* is often isolated from diseased and dead insects; Bucher classified it as a facultative pathogen. It produces a phospholipase (4, 28, 48, 54). Although it is recorded as being frequently associated with natural populations of grasshoppers and locusts, and some 75 insect hosts are known, it is more commonly isolated from insects reared under laboratory conditions than from insects taken from the field. Red and nonchromogenic strains have been isolated (79, 80, 83).

The use of such nonsporulating bacterial pathogens of insects is beset with some difficulties, among which are greater sensitivity to drying and sunlight, and a greater tendency to variability of virulence. *Pseudomonas* spp. and *Serratia* spp. also have the disadvantage that they include strains demonstrating some degree of pathogenicity for mammals (5, 9, 10, 84). However, the nonsporulating bacteria are responsible for much mortality under natural conditions, and it is possible that as we add to our knowledge we will be enabled to circumvent some of their shortcomings. Research is required to achieve a better understanding of the metabolism, in insects, of such types of bacteria, and particularly of their oxygen requirements and the kinds of proteolytic enzymes they produce. As Bucher pointed out, the relatively anaerobic conditions of the insect gut may inhibit or limit growth of such bacteria and, thus, the subsequent production of lytic enzymes that lead to successful invasion of the hemocoele (16).

SPOREFORMING BACTERIAL PATHOGENS

The sporeforming bacteria of the family Bacillaceae comprise the greater part of the known bacterial pathogens that have been seriously considered for use as microbial insecticides.

Clostridium Species

Species of the genus *Clostridium* have been isolated only rarely from diseased insects. It

should be noted, however, that the procedures most often used in the past would not favor the growth of anaerobic bacteria. In 1957, Bucher (13) isolated two obligate anaerobes, which he named *C. brevifaciens* and *C. malacosomae*, from diseased larvae of *Malacosoma pluviale* (Dyar), the western tent caterpillar. If tent caterpillar larvae are fed spores of *C. brevifaciens*, germination occurs and abundant multiplication takes place in the mid-gut. At about 72 hr, larvae demonstrate a characteristic accordionlike shortening and eventually die; Bucher called this condition "brachyosis." *C. malacosomae* produces similar symptoms. A medium has been developed that will support vegetative growth but not sporulation of these species (17).

Another *Clostridium* species quite similar to the tent caterpillar pathogen has been found in the Essex skipper *Thymelicus lineola* (Ochsenheimer) (21). Atger (6) reported that a *Clostridium* species is involved in an epidemic disease of the pine processionary moth. Thus, it appears that if we apply the proper screening methods we may discover other anaerobic pathogens, and it is conceivable that other potentially useful anaerobes still await discovery.

Although Bucher showed that it is possible to induce disease, under field conditions, by introduction of spores of *C. brevifaciens* and *C. malacosomae* into the tents or the feeding areas of young larvae of *Malacosoma americanum*, the eastern tent caterpillar, the fact that *C. brevifaciens* will not sporulate on artificial media limits production of spore suspensions on a commercial scale (17). Since in some parts of North America *Malacosoma* spp. often require control measures, solution of this difficulty might present new opportunities to exploit this group of bacteria.

Bacillus Species

Several sporeforming aerobes have been tested for suitability as microbial insecticides, among which are *B. cereus* Frankland and Frankland, *B. thuringiensis* Berliner, and *B. popilliae* Dutky. The role of these as insect pathogens has been discussed in considerable detail in a number of recent reviews (3, 27, 42, 44, 45). *B. popilliae*, which causes type A milky disease in larvae of the Japanese beetle *Popillia japonica* Newman, has been widely used as a successful biological control agent (27). It is the subject of a separate paper in this symposium (59).

B. cereus. *B. cereus*, a ubiquitous soil form, has often been isolated from diseased and dead larvae under its own name, and under numerous misnomers. Although most of the isolates are probably potential pathogens, a few are known to be facultative pathogens and characteristically grow quickly and abundantly on simple media. A number of varieties are pathogenic when ingested, and susceptible hosts are found in several orders of insects. Stephens isolated several such strains from diseased codling-moth larvae (74, 75). Heimpel also found this organism in diseased larch sawfly larvae (35, 36, 37, 38), and Smirnoff isolated a variety pathogenic for the spruce budworm (63). All of these have been tested on a limited scale under field conditions; they caused some mortality but not to an extent that justified further work.

Characteristically, these *B. cereus* isolates are from insects whose gut conditions permit abundant growth and the elaboration of enzymes such as phospholipase C. Heimpel (37) showed that this enzyme is an important factor in the larch sawfly disease because it causes damage to the gut cells of the insect. There has been a tendency to abandon work with *B. cereus* in favor of the so-called crystalliferous bacteria, since they possess most of the attributes of *B. cereus* plus some others. [The literature up to 1959 on the crystalliferous bacteria has been reviewed in detail in an earlier issue of this journal (59). A very large number of titles have since appeared, but only a few of them are cited here.]

B. thuringiensis. The term "crystalliferous" has been applied to a number of *Bacillus* spp. which, in addition to the endospore, produce a discrete characteristic inclusion in the sporulating cell; these are known as parasporal inclusions or parasporal bodies, and popularly as crystals (34). A mature or sporulated culture normally contains both spores and the crystals, but under some conditions cultures can become acrystalliferous or asporogenous, or both (41, 61, 62). A number of crystalliferous species have been found associated with insects; the best known are varieties of *B. thuringiensis* Berliner, which is closely related to *B. cereus* and shares with it most of its biochemical, morphological, and metabolic characters (40). The Mattes strain of *B. thuringiensis* var. *thuringiensis* is the basis for a number of commercial preparations produced in the United States and in several European countries (11). The effectiveness of such preparations is illustrated in Fig. 1. These preparations meet to a considerable degree the criteria mentioned earlier; consistent virulence, persistence, speed of action, specificity, safety of use, and ease of production.

Historically, *B. thuringiensis* and strains related to it have been isolated and field-tested with varying success on a number of occasions since the beginning of the century (71). Beginning about 1950, a number of workers, European and

FIG. 1. *Effectiveness of a commercial preparation of Bacillus thuringiensis (Thuricide; 2 lb per 100 gal of water) in preventing defoliation of apple trees by larvae of the winter moth, Operophtera brumata Linnaeus. The control (left) was unsprayed. Illustration courtesy of R. P. Jaques (43).*

North American, reported findings that collectively have greatly aided the rational use of this kind of pathogen (41, 42, 44).

It has been found that there are numerous varieties of crystalliferous *Bacillus* spp. associated with Lepidoptera larvae (see Table 1). The available evidence suggests that there is perhaps a degree of adaptation to particular insect host species. Thus, *B. thuringiensis* var. *sotto* Ishiwata, originally isolated from the silkworm, is much more toxic for this insect than is *B. thuringiensis* var. *thuringiensis*, which Mattes originally isolated from the flour moth (2, 44, 47, 70). Vankova (85) tested 12 different strains of crystalliferous bacteria against eight different Lepidoptera, and found evidence of selective toxicity. Grigorova (31) presented evidence to indicate that strains of *B. thuringiensis* isolated from the gypsy moth, *Lymantria dispar* L., are more pathogenic for this insect than are strains that appear identical in biochemical and serological tests but that were isolated originally from other insect species. Krieg and Franz (46) isolated from the waxmoth, *Galleria mellonella* L., a strain which is highly virulent for this insect; it is biochemically indistinguishable from the Mattes flour moth isolate, but the latter is only weakly virulent for the waxmoth.

The similarity of *B. thuringiensis* to the catch-all description for *B. cereus* (which is a group and not a discrete species in the strictest sense) has led some to question as to whether *B. thuringiensis* is a valid taxon (8, 42, 44). In spite of this, insect pathologists in general have continued to use the epithet *thuringiensis*, partly on the grounds of convenience. A large number of isolates have been studied by a variety of methods (41, 55). De Barjac and Bonnefoi (7, 8) determined the cultural and biochemical characters of 50 strains of the *B. thuringiensis* group. They also used the method H-antigen analysis and were able to identify nine distinct serotypes (Table 2). Norris (56, 57) subjected many of the same strains to electrophoretic analysis of the esterase enzyme systems present in extracts of vegetative cells. The esterase patterns closely parallel the divisions arrived at by means of H-antigen analysis, with minor exceptions. Antigenically, *sotto* and *dendrolimus* serotypes are identical but they possess different esterase patterns.

Taken as a whole, these studies have been most fruitful and indicate that between strains there are consistent biochemical and serological differences that can be correlated with host specificity and relative virulence. This work, and the occasional isolation of new strains from new hosts, suggest that *B. thuringiensis* is a widespread and perhaps cosmopolitan complex or group (1, 7, 8, 57).

In the past 10 years, a number of toxic mechanisms or entities have been demonstrated in *B. thuringiensis* varieties (22, 24, 42). These include: the enzyme phospholipase C, the proteinaceous parasporal inclusions (crystals), heat-stable exotoxins, and heat-labile exotoxins. If spores or vegetative cells of *B. thuringiensis* are injected into the hemocoele of an insect, abundant growth soon takes place, and this leads quickly to a fatal septicemia. The fate of spores or cells taken by mouth depends on gut conditions, including *p*H, oxidation-reduction potential, degree of anaerobiosis, antibiotics of plant origin, the digestive enzymes of the host, and other factors.

TABLE 1. *Occurrence of crystalliferous bacteria in various species of insects**

Bacillus species and variety†	Host	Culture designation	Source
B. thuringiensis			
var. *thuringiensis*	*Anagasta kühniella* (Zell.)	996	N. R. Smith, USA
var. *thuringiensis*	*Heliothis obsoleta* (F.)	H-III	S. Majumder, India
var. *thuringiensis*	*Pristiphora erichsonii* (Htg.)	Smirnoff	W. Smirnoff, Canada
var. *thuringiensis*	*Galleria mellonella* (L.)	galleriae	A. Krieg, Germany
var. *thuringiensis*	*Psylla pyricola* (Först.)	DD-749	A. Heimpel, USA
var. *thuringiensis*	*A. kühniella*	E-1	J. Norris, England
var. *thuringiensis*	*Melolontha melolontha* (L.)	LBG-B4058/c	L. Ettlinger, Switzerland
var. *thuringiensis*	*Eisenia foetidus* (Sav.) earthworm	EW-1; EW-2	A. Heimpel, USA
var. *thuringiensis*	*Ephestia elutella* (Hbn.)	Epc 2000	S. Dutky, USA
var. *thuringiensis*	*Plodia interpunctella* (Hbn.)	Va	C. Vankova, Czechoslovakia
var. *alesti*	*Bombyx mori* L.	alesti	C. Toumanoff, France
var. *alesti*	*B. mori* litter	anduze	C. Vago, France
var. *alesti*	*Euxoa segetum* Schiff.	euxoae	A. Krieg, Germany
var. *alesti*	*A. kühniella*	K-17; K-18	E. Karstak, France
var. *sotto*	*B. mori*	sotto	M. Ono, Japan
var. *sotto*	*Dendrolimus sibericus* Tshkv.	dendrolimus	E. Talalaev, Russia
var. *sotto*	*Trichophaga tapetzella* (L.)	P.I.L. 94	G. Ayerst, England
var. *galleriae*	*G. mellonella*	G-1	J. Norris, England
var. *galleriae*	*P. interpunctella*	P.I.L. 106	J. Norris, England
var. *variabilis*	*B. mori* litter	T63-L4	K. Aizawa, Japan
var. *variabilis*	*Heliothis assulta* (Guen.)	HA-3	K. Aizawa, Japan
var. *variabilis*	*E. elutella*	IH-A	K. Aizawa, Japan
var. *variabilis*	*P. interpunctella*	58-3-1	E. Steinhaus, USA
var. ?	*P. interpunctella*	DD-788	M. Day, Australia
var. ?	*A. kühniella*	DD-742	I. Hall, USA
var. ?	*G. mellonella*	G-2	J. Norris, England
var. ?	*P. interpunctella*	Tolworth	J. Norris, England
B. entomocidus			
var. *entomocidus*	*Aphomia gularis* Zeller	EAS57-1-1	E. Steinhaus, USA
var. *subtoxicus*	*P. interpunctella*	EAS58-1-1	E. Steinhaus, USA
B. finitimus	*Malacosoma disstria* (Hbn.)	Ma-d-7000	A. Heimpel, USA

* From data kindly supplied by A. M. Heimpel.
† Nomenclature as suggested by Heimpel and Angus (40).

The enzyme phospholipase C, which is involved in the pathogenicity of *B. cereus* for the larch sawfly (42), has also been demonstrated in *B. thuringiensis* varieties, and presumably is operative if produced. For it to be produced, there must be vegetative multiplication, and this depends on the successful establishment of the pathogen in the insect gut. In such circumstances, additional hydrolytic enzymes, such as proteinases and carbohydrases, are also produced, but few of them have been isolated and identified.

The importance of the parasporal inclusions is that the protein that comprises them appears to act as a protoxin or toxin, damaging the mid-gut cells of susceptible species in such a way as to inhibit feeding, and causing other changes favoring growth of the pathogen (42). The susceptible Lepidoptera include species affected by either the crystal alone or the spores alone. There is a gradation of response, and in most species the greatest mortality is caused by preparations which are a mixture of these entities.

Laboratory and field tests indicate a wide host range for some varieties: serotype *berliner*, 147 species of insects in laboratory and 97 in field tests; serotype *sotto-dendrolimus*, 28 and 7; and serotype *alesti*, 42 and 14, respectively (compiled from information kindly supplied by the Centre de Documentation Bibliographique of the Commission Internationale de Lutte Biologique Contre les Ennemis des Cultures). The toxicity of the crystal protein seems to be limited to Lepidoptera.

In ingestion tests with larvae of the silkworm, *Bombyx mori* L., it has been found that a dose of 0.05 μg of crystals per g of insect will induce

TABLE 2. *Classification of Bacillus thuringiensis serotypes on the basis of H antigens**

Serotype	Common name
1	berliner
2	finitimus
3	alesti
4 a, b	sotto, dendrolimus
4 a, c	kenyae (PIL 94)
5	galleriae
6	subtoxicus, entomocidus
7	aizawai
8	morrisoni

* Bonnefoi and de Barjac (8).

paralysis in 2 hr. The values obtained with spore-crystal mixtures are proportional to the crystal content of the mixture. Similar values have been obtained for other species of Lepidoptera; in these instances, septicemia is induced in 12 to 48 hr (Angus, *unpublished data*).

The phospholipase and the parasporal toxin are nondialyzable, heat-sensitive proteins. A number of water-soluble fractions of unknown composition, which are toxic for some insects, have been isolated from cultures of *B. thuringiensis* (23, 25, 41, 44, 64). Some are toxic by ingestion, some only by injection; both heat-labile and heat-stable fractions have been reported. They are quite unlike either the phospholipase or the parasporal protein in their action on insects, and some have been found to be active against some Lepidoptera, Coleoptera, Orthoptera, Hymenoptera, and Diptera species. The importance of these as yet uncharacterized toxic fractions is that they extend the host range of *B. thuringiensis* to a wider range of insects, some of which are pests of medical significance. It is known that the occurrence, concentration, and perhaps behavior of these fractions is dependent on the strain of bacteria used, the medium, culturing conditions, and the method of harvesting or concentrating the final product. A product based on the residue obtained after removal of fluid by centrifugation will obviously be less rich in water-soluble compounds than a product prepared by an evaporation technique. It would seem that the most potent product could be achieved by first splitting the starting material into several fractions, processing these appropriately, and combining them to obtain a final product.

Little or nothing is known of the specific mode of action of the water-soluble toxins. Indeed, the mode of action of the parasporal protein is only imperfectly understood. On the basis of past experience, it appears that more detailed knowledge of all of the toxic components will aid rational exploitation of the crystalliferous bacteria. The fact that strains of this kind of bacteria do vary in their specificity and behavior increases the opportunities to counter the flexibility of insects with that of another living form.

Insofar as bacteria are concerned, the century-old idea of biological control of insects has come nearest to fruition with *B. popilliae* and *B. thuringiensis*. The difficulty of producing spores of the milky-disease organism in a completely synthetic process constitutes a drawback. The attention that is being given this problem by various research groups indicates that, once this difficulty is overcome, increased use will follow. The crystalliferous bacteria are more easily produced in a virulent state, but their best use is hindered by our incomplete knowledge of this group. As our knowledge of strain differences increases it is probable that we will be able to use them more effectively. There would certainly seem to be more than sufficient grounds for making the attempt.

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